Biological Monitoring of Dichloropropene: Air Concentrations, Urinary Metabolite, and Renal Enzyme Excretion

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ABSTRACT. Fifteen applicators of dichloropropene (DCP) were studied for personal air exposure to DCP, excretion of the metabolite of DCP (3CNAC), and excretion of the renal tubular enzyme, N-acetyl glucosaminidase (NAG). Each was studied for four 6–8 h consecutive intervals following baseline determinations of 3CNAC and NAG excretion. In accord with pilot data, 24-h urinary excretion of 3CNAC (mg) correlated well with exposure product for DCP (min exposed · mg/m³), $r = 0.854$, $p < .001$. A more precise correlation of the air exposure product with urinary excretion of 3CNAC was discerned by using the morning urine after the previous day of exposure (μg/mg of creatinine), $r = 0.914$, $p < .001$. Four workers had clinically elevated activity of NAG (> 4 mU/mg creatinine) in any of their urine collections after baseline. Nine workers showed greater than 25% increases in NAG excretion when compared to baseline. Dichloropropene air exposure products of > 700 mg · min/m³ or excretion of > 1.5 mg 3CNAC/d distinguished abnormally high daily excretion of NAG. These data demonstrate a firm positive relationship between air exposure and internal exposure, and a possible subclinical nephrotoxic effect in DCP workers.

CIS- AND TRANS-1,3-DICHLOROPROPENE (DCP) are the components in commercial soil fumigants marketed as Telone (Dow) and previously as DD-soil fumigant (Shell). Nematodes in soil that are harmful to the roots of commercial agricultural crops (e.g., potatoes, berry fruits, crucifers, citrus) are eliminated with use of DCP. Because ethylene dibromide and dibromochloropropane were found to be carcinogens and reproductive hazards, DCP applications have increased to over 14 million lb/y (6.5 million kg/y) in California. The DCP is pumped into the soil through steel shanks (chisel injection) that are pressed a few inches into the earth when pulled behind a tractor. Applicators are exposed to DCP during operations that include transfer of DCP from storage tanks into the tanks on the tractor and during repair/cleaning operations. Also, it has been demonstrated that applicators and bystanders are exposed to airborne DCP when the steel shanks are lifted

from the soil at the end of each tilled row and to volatilized DCP (vapor pressure 28 torr) from the soil itself.[1,2] Workers exposed to airborne DCP at concentrations of 1.5 mg/m³ will excrete significant quantities (3–8 mg) of the metabolite, N-acetyl-S-[cis-3-chloroprop-2-enyl]-cysteine (3CNAC), in their urine during and following field applications.[2,3] We have also shown that the 24-h urinary excretion of 3CNAC will increase in approximate proportion to the DCP exposure product (air concentration × minutes of exposure).

Whereas both external and internal exposure are apparent, little investigation on dosimetry or adverse effects has been performed. Title III of the California Administrative Code (August 1985, Section 6468(d) (2)) states that employees should be warned that DCP causes kidney damage. However, neither monitoring of renal function is performed nor has research attempted to establish dose-effect relationships. Subclinical injury could go unnoticed.

DCP is an indirect mutagen[4–8] that binds[5,6] and depletes sulfhydryl groups in the liver and kidney.[6] In vitro microsomal experiments demonstrate that sufficient added glutathione will prevent mutagenicity in S9 activated Ames assays.[5,6] Earlier studies have indicated that the rat kidney may show toxic effects in the renal tubular epithelium following chronic inhalation of concentrations as low as 3 ppm.[10] This effect has not been confirmed in two subsequent subchronic animal studies following inhalation of concentrations as high as 150 ppm.[11,12] However, one of these investigations has shown that only a 3-h exposure to 90 ppm will decrease the sulfhydryl content of the kidney and liver by 31% and 39%, respectively.[9] Acute studies with higher doses have demonstrated kidney and liver necrosis.[10,13] As alternatives to kidney histology, other sensitive measures of renal injury have not been applied, such as N-acetyl glucosaminidase (NAG) release from the kidney tubule.[14]

In a recent pilot study, we showed that two of three applicators had elevated NAG excretion. The current study investigates the dosimetry between DCP air exposure, 3CNAC metabolite excretion, and NAG as a measure of subtle nephrotoxicity.

Methods

Fifteen male applicators (11 workers evaluated 1 d each, 4 of these studied on a second application) were studied under the authority of the Worker Health and Safety Branch of the California Department of Food and Agriculture (CDFA). Applicators were chosen on a first available basis in the locale where DCP was applied. Each applicator completed a short questionnaire that recorded current medications and current and past medical illnesses. Twelve of the applicators were studied at least 40 h after any previous application of pesticides in an attempt to preclude exposure from a previous work day. The study period for each applicator consisted of five consecutive intervals. The first interval was a pre-exposure, morning urine collection. Each of the second through fifth intervals was 6–8 h in duration. Exposure and personal air sampling occurred in the second (morning) and sometimes the third interval (afternoon). Post-exposure intervals (fourth and fifth) corresponded with evening and overnight periods, respectively. The exposure periods for the various applicators ranged from 2–7 h. Workers wore long-sleeve coveralls during application of DCP. During loading, workers wore chemical-resistant gloves, aprons, boots, and NIOSH-approved respirators. Dermal exposure from small spills during these closed loading operations may have occurred, but none were observed. Field application rates were 15–68 l (4–18 gal) per acre. Soil was free of plant debris, dry, and 12.8–18.3 °C (55–65 °F). Air temperatures were between 21.1–32.2 °C (70–90 °F) and winds were less than 10 mph.

DCP air monitoring. At the field site, prior to beginning work, the applicator was attached with a personal air sampling device and instructed as to collection of urinary specimens. Operator breathing zone air samples were drawn by personal air sampling pumps (MSA C210) via tygon tubing connected to charcoal adsorbent tubes (SKC #226-09, 400/200 mg). Tube openings were position down across the chest and did not interfere with work activity. Sorbent tubes were changed at up to 4 h or when there was an obvious change in work practice (loading vs. repair) or intervals. These tubes were capped and stored on dry ice, then refrigerated (−20 °C) until analysis. Amounts of DCP in sorbent tubes were determined in accord with established procedures[15,16] that included elution of tubes with carbon disulfide mixed with an internal standard and direct injection onto an electron capture gas chromatograph. Sensitivity and precision of these determinations is 0.1 μg per collection (column). Exposure to DCP in air is expressed as the exposure product, i.e., the concentration of DCP in mg/m³ multiplied by the minutes of exposure.

Urine collection. Urines were collected by instructing the applicators to urinate into separate 500-ml opaque polyethylene containers for each voiding. Each worker voided into a single container until the end of the interval. At this time, each worker was asked to void again. Urine collections coincided with the end of an interval and a change in air monitoring tubes. Each void was mixed and the volume was measured and aliquoted into 50-ml polyethylene screw cap containers. These were stored on dry ice for the day of collection and at −70 °C until analysis. Completeness of urine collections was assessed by total creatinine excretion and by direct observation (during work hours). All 24-h urine collections contained more than 1 g of creatinine. Average urine volume per interval was 271 ± 153 ml.

Urinary 3CNAC determinations. The syntheses of the major metabolite, N-acetyl-S-(cis-3-chloroprop-2-enyl)-cysteine (3CNAC), and the isomeric internal standard, N-acetyl-S-(2-chloroprop-2-enyl)-cysteine (2CNAC), from N-acetyl cysteine and the appropriate dichloropropene have been described in our previous work.[2,3] Determination of 3CNAC in urine was performed by an improved gas chromatographic-mass spectrometric (GC-MS) procedure as follows. Frozen urine is thawed to room temperature and 1.0 ml is mixed with 0.1 ml of 6 N HCl and 10 μl of the stock internal standard

(2CNAC, 1 mg/ml) in a 13 × 100 mm screw top borosilicate tube. Standards are prepared by spiking urine with appropriate quantities of the stock 3CNAC methanolic solution. Standard concentrations were 0, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 μg/ml of urine. Two ml of *t*-butyl methyl ether (TBME) is added to each tube, capped, and extracted for 10 min while shaking. All samples are centrifuged for 10 min at 2000 *g*. The TBME phase (1.5 ml) is transferred to a new labeled tube and evaporated under nitrogen at 40 °C. In an ice bath, 200 μl of an ether solution of diazomethane is added to the residue at 5 °C and capped. Following a reaction time of 30 min, the ether and excess diazomethane are evaporated to dryness under nitrogen flow at room temperature. Two ml of n-butyl chloride is added, mixed, followed by 1 ml of water. This extraction/wash is mixed by vortex for 10 s and then centrifuged for 10 min at 2000 *g*. The n-butyl chloride (1.5 ml) is evaporated under nitrogen at room temperature. Toluene (300–600 μl) is added to the residue for GC-MS analysis.

Capillary GC-MS instrumentation included a Hewlett-Packard 5970B quadrapole mass selective detector connected to a 5890 gas chromatograph. The fused silica column was 12 m × 0.2 mm and coated with cross-linked methylsilicone. Helium carrier gas flow was 1 ml/min. The injector was heated to 200 °C and operated in the splitless mode. The column was heated at 70 °C for 4 min, then increased at 20 °C/min to 225 °C for a total program time of 12 min. The quadrapole was calibrated with perfluorotributylamine at masses of 69, 219, and 502. The electron multiplier voltage was 1 600 volts and ionization voltage was 70 keV. Mass peak widths at half-height (resolution) were 0.5 atomic mass units. The ions monitored were 117 and 176 with a dwell time of 50 ms each. One μl of the toluene sample was injected by a Hewlett-Packard 7673 automatic sampler. After standards calibration, all samples were determined in the same run. Quantitation was performed by comparing peak height ratios of 3CNAC methyl ester/2CNAC methyl ester for the ion monitored (either 117 or 176) to a standard curve of peak height ratios for the standards. The assay was linear from 0–30 μg/ml with a sensitivity of 0.1 μg/ml with the 117 ion. Intrassay coefficients of variation are 4–8%. Quality control samples (spiked urines, 0.5 and 10 μg/ml) were extracted with other samples in batch and injected after every 10 sample determinations. Both trans-3CNAC and cis-3CNAC can be measured in this assay. Only cis-3CNAC is determined since earlier animal studies indicated a higher percentage conversion to cis-3CNAC.[22]

Urinary NAG determinations. Urinary activities of NAG were determined by the manual method of Powell et al.[17] with modifications incorporated by Yu and Osterloh.[18] This is a fluorometric method that will measure the hydrolysis of 4-methylumbelliferyl-Beta-D-glucosaminide. Enzyme activity in nanomoles/min (milliunits, mU) was expressed in terms of milligrams of creatinine excreted (mU/mg creatinine), since this corrects for variability due to urine flow rates.[19] The interassay coefficient of variation was 2.7% at 2.5 mU/mg creatinine. Quality control samples (1 and 4 mU/mg) were performed after every 10 determinations in batch runs. The normal reference interval in humans is < 4 mU/mg of creatinine.[17] The 24-h excretion of NAG was expressed as the total units of activity excreted during the 24 h after the onset of exposure. Creatinine concentrations were determined by the Jaffe reaction.[20]

Statistical analysis. Correlations between 3CNAC excretion and DCP exposure products or NAG excretion were performed using Pearson's correlation. Dichotomous comparisons between NAG excretion and 3CNAC excretion or DCP exposure products were performed with Mann Whitney U test or Student's *t* test.

Results

Descriptive data. Table 1 shows the means, medians, range, and standard deviations for the measures of DCP exposure and excretion of 3CNAC and NAG. Figure 1 gives the data for two workers by interval for a short, single exposure and an exposure to lower concentrations of DCP, but for a longer time.

DCP air exposure. Air concentrations ranged from 0.26–9.39 mg/m³ (Table 1). Duration of exposure ranged from 120–697 min. Six workers were exposed during the second interval only (morning interval) and eight workers were exposed during the second and third interval (similar to worker B in Fig. 1). The DCP air exposure product ranged from 62–3 780 mg · min/m³. Air monitoring was not collected uniformly in one applicator and therefore was excluded from later comparisons of DCP exposure product to 3CNAC excretion or NAG excretion.

Urinary 3CNAC excretion. The excretion of 3CNAC during the pre-exposure (first) interval was less than 0.07 mg in 12 of the 15 applicators. Three other workers showed amounts in this first urine collection of 3.96 mg, 0.52 mg, and 0.30 mg. Two of these 3 with elevated baseline excretion were from 3 workers known to be exposed on previous days. The 24-h urinary excretion of 3CNAC (following baseline collection to next morning) averaged 2.58 mg and varied from 0.50–9.17 mg. The occurrence of peak 3CNAC concentrations in urine occurred in the interval immediately following the exposure interval in 5 of 6 workers with single interval exposures (Fig. 1). In 6 of 8 of those applicators with double interval (second and third intervals) air exposures, peak 3CNAC concentrations occurred during the third interval. One worker analyzed during two applications showed late peak concentrations in both instances.

Urinary NAG enzyme. The mean excretion of NAG for all intervals was 2.63 mU/mg of creatinine. The occurrence of a urine collection with greater than an abnormal amount of NAG (clinically abnormal > 4 mU/mg creatinine) for any applicator was observed in 4 of the 15 workers. The occurrence of increases in the excretion of NAG activity that were 25% greater than baseline occurred in 9 of the 15 workers over any of the four intervals. Decreases of more than 25% occurred in 4 workers. The range of intraindividual variability in NAG excretion after correcting for creatinine is less than 25%.[19,21]

Table 1.—Descriptive Data: Exposure to DCP and Excretion of 3CNAC and NAG

Interval exposure and excretion	N	Mean	Deviation	Standard range	Median
Air concentrations of DCP (mg/m³)	22	2.56	2.40	0.26- 9.39	1.70
Excretion of 3CNAC (μg/ml)	73	2.70	4.77	0.18-33.02	1.06
Excretion of 3CNAC (mg/interval)	73	0.69	1.14	0.02- 7.00	0.27
Excretion of 3CNAC (μg/mgC)*	74	1.37	1.86	0.07- 8.58	0.69
Excretion of NAG (mU/ml)	74	5.08	3.58	1.00-18.00	4.00
Excretion of NAG (mU/mgC)*	74	2.63	1.21	1.00- 7.67	2.34
Excretion of NAG (mU/interval)	74	1 196	881	113-5 331	1 022
24-h exposure and excretion					
Time exposed to DCP (min)	14	424	228	120- 697	435
DCP exposure products (min · mg/m³)	14	990	916	62-3 780	852
Excretion of 3CNAC (mg/24 h)	15	2.58	2.45	0.50-9.17	1.39
Excretion of NAG (mU/24 h)	15	4 940	2 291	278-8 956	4 068

*mgC = per mg of creatinine in urine.

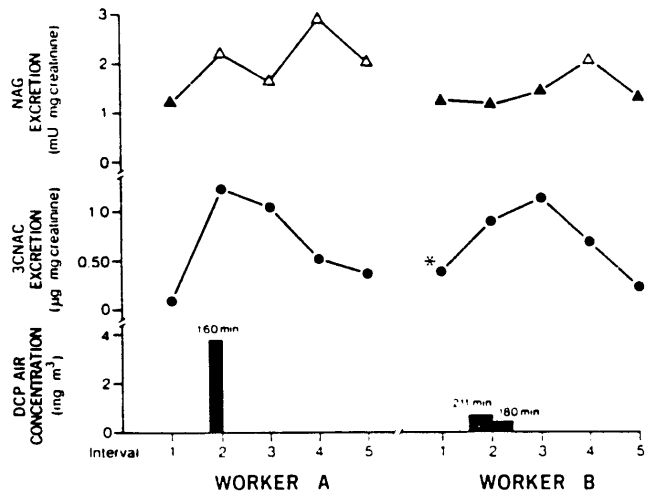

Fig. 1. Exposure and excretion data for a worker with a single interval of exposure to DCP(A) and a worker with a two-interval exposure to lower concentrations of DCP(B). Open triangles (Δ) represent NAG excretion, which is greater than 25% over baseline. * = Worker B, who probably had residual exposure.

Data relationships. The various modes of expressing urinary excretion of 3CNAC and their correlation with the DCP air exposure products are shown in Table 2. The 24-h excretion of 3CNAC correlated well with DCP air exposure product, as in previous work. The 3CNAC excretion in the urine of the following morning (μg/mg creatinine) had a more precise correlation with the DCP air exposure product. The cumulative daily excretion of NAG showed significant positive relationships with the various measures of 3CNAC excretion (see measures listed in Table 1) and to daily air exposure product (range of r = 0.483-0.520). For example, the relationship between the 3CNAC in the next morning urine (μg/mg of creatinine) and the 24-h excretion of NAG was positively correlated (r = 0.514, Fig. 2). This data was also analyzed dichotomously (Fig. 3). For daily urine excretions of 3CNAC that were greater than 1.5 mg/day, higher mean amounts of NAG (6.74 vs. 3.37 × 10^3 mU/24 h) are clearly excreted in those workers exceeding this measure of internal dose (p = .001). This was similarly true for daily air exposure products exceeding 700 mg · min/m³ (p < .005), NAG excretion being 6.43 vs. 3.17 × 10^3 mU/24 h, respectively.

Other dichotomous relationships and correlations existed within this data when considering only the interval data as opposed to the cumulative daily time periods. For example, the excretion of more or less than 0.5 mg of 3CNAC per interval was associated with differences in exposure products per interval (p < .001, N = 71) and with the differences in NAG excretion (p < .001, N = 71). Weaker relationships across intervals might be expected because peak urinary excretion was found not to occur in the same interval as peak air exposure or peak NAG excretion. These would perhaps correlate better if intervals could be staggered. At this time, however, there is insufficient information on which to base a staggered design.

Discussion

We have demonstrated that 3CNAC is excreted in relation to external air exposure of DCP. This finding in these applicators confirms and extends the preliminary data from two earlier small studies.[2,3] Because the regression for 3CNAC excretion vs. DCP exposure product is reasonably precise given interindividual variation, we believe this attests to pulmonary exposure as being the predominant route. However, dermal exposure could play a significant role during contact with spills. There is a paucity of animal data available for interpretation. The actual contribution of dermal exposure is unknown in human workers.

Climie et al.[22] and Huston et al.[23] had used ^{14}C-labelled DCP to show that excretion of urinary cis-3CNAC ac-

counted for 70% of the dose in oral gavage studies in rats. After recognizing the potential for its use as marker of exposure, we had synthesized unlabelled 3CNAC and appropriate internal standards and developed a GC-MS method for determining 3CNAC in human urine.[2] Initial applications to the urine of applicators revealed large quantities of the metabolite in spot urines (5.2–27.0 μg/ml), with daily cumulative excretion of 3CNAC in rough proportion to DCP exposure product. This rough relationship was again observed in a study of three workers over 2–3 d.[3] Also, substantiation that 3CNAC excretion is an appropriate biological monitoring technique is derived from qualitative differences in excretion that resulted from differences in field application rates. In this study, application rates were one-half that of the pilot study.[3] Similarly, average 24-h 3CNAC excretion was approximately one-half, 5.82 ± 3.98 mg (N = 5) vs. 2.58 ± 2.45 mg in the current study.

We also had observed that excretion of peak concentrations (or amounts) in two workers followed peak DCP air exposure by as long as 16 h. The present study confirms this also with peak excretion within two intervals (< 16 h) after exposure. Accumulation of 3CNAC from day to day may be slight, because measurable amounts are still present on the morning following exposure. Three of the workers showed elevated amounts of 3CNAC in their baseline urine collections indicating either exposure earlier that day, on the prior day, or slower elimination. Two of these three workers were suspected to have exposure on the prior day.

In the previous pilot study, urinary concentrations of 3CNAC with and without correction for creatinine excretion showed a poorer correlation with DCP air exposure product than did amounts (mg) of 3CNAC excreted. Presently, the concentration of 3CNAC in the morning urine following exposure showed an even more precise correlation than 24-h 3CNAC excretion. This may have been a more precise indication of exposure because the influence of the variability in absorption, distribution, and metabolism are more attenuated at this later time, whereas analytic precision may remain constant. Using morning-after urine may be a useful, more practical technique to the biological monitoring of DCP exposure.

Protection and biological monitoring of workers is of concern because DCP has produced forestomach squamous and urinary bladder transitional cell cancers following chronic oral gavage studies in rats and mice.[24] Subchronic inhalation studies in rats and mice at 90 or 150 ppm have demonstrated hyperplasia of the respiratory epithelium, degeneration of nasal mucosa, and hyperplasia of transitional cell epithelium in the urinary bladder (W. T. Scott, Dow Chemical Co., personal communication). Chronic inhalation studies are not published, but increased respiratory neoplasia are suggested (W. T. Scott, personal communication). Be-

Table 2.—Relationships between Air Exposure Product and 3CNAC Excretion

Air exposure product versus:	r	p
24-h 3CNAC excretion (mg)	0.854	<.001
Peak 3CNAC excretion interval + following interval (mg)	0.847	<.001
Excretion in two intervals after peak exposure (mg)	0.694	<.01
Morning after exposure (μg/mg of creatinine)	0.914	<.001
Morning after exposure (μg/ml)	0.843	<.005

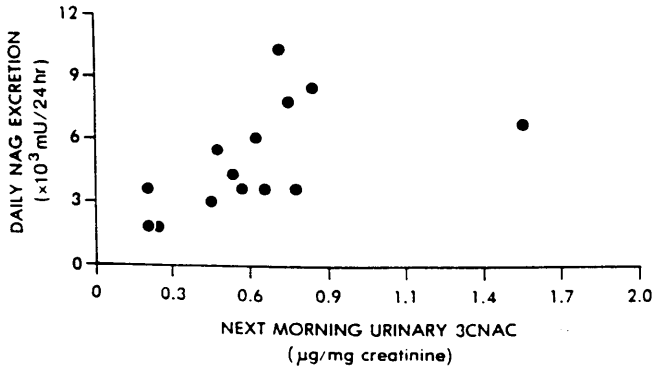

Fig. 2. Correlation of daily cumulative NAG excretion (× 10^3 mU/24 h) vs. the amount of 3CNAC excreted in the urine of the next morning (μg/mg creatinine), r = 0.514.

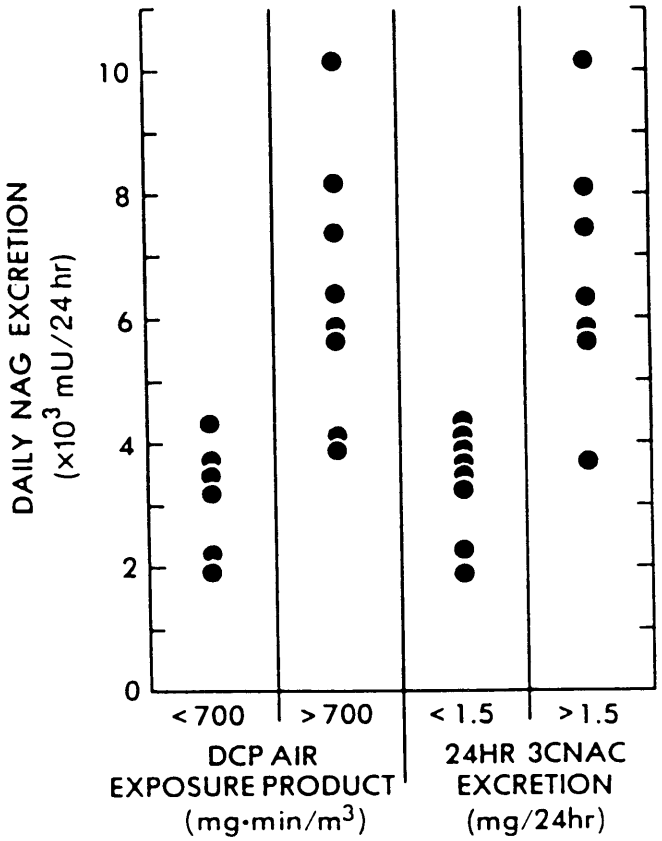

Fig. 3. Daily cumulative urinary excretion of NAG (× 10^3 mU/24 h) as a function of thresholds for DCP air exposure product (mg min/m³) or daily cumulative excretion of 3CNAC (mg/24 h).

cause of its possible carcinogenesis in animals, the Environmental Protection Agency (EPA) had placed DCP under Special Review (EPA Case #65-0328 and Fed Reg 51:36160-2, Oct. 8, 1986).

In addition to protecting workers from the possible carcinogenic effect of DCP, protection from nephrotoxic effects is also of concern. Whereas the animal data are suggestive but conflicting with regard to toxic dose,[10-13] methods for determining nephrotoxicity have relied primarily on histology. A recent study by Stott and Kastl showed an early subclinical and potentially nephrotoxic effect, the decrease in thiol content of the rat kidneys following a 3-h inhalation at 90 ppm (405 mg/m³). Thiols are probably protective toward DCP-mediated toxicity. Added thiols (glutathione) preclude S9 mediated Ames mutagenicity.[5,6] The N-acetyl cysteine conjugate of DCP (a mercapturic acid) is a result of glutathione conjugation with a reactive metabolite of DCP.[22,23] Cis-1,3-dichloropropene oxide (an epoxide) is a possible reactive intermediate.[5,25] Insufficient amounts of cellular thiols would allow this reactive intermediate to react with other cellular components to result in cell dysfunction and necrosis. Thiol stores or status may explain why not all similar exposures would produce exactly similar responses of NAG release. In contrast, the conjugated metabolite itself may cause nephrotoxicity. Recently, 1,2-dichlorovinyl cysteine and glutathione conjugates have been shown to be direct potent nephrotoxins.[26] Because of the similarities in structure, 3CNAC might also produce this type of injury. Thus, nephrotoxicity may be mediated through a microsomally generated reactive metabolite or mediated via the glutathione conjugate or both. The former mechanism follows the models of carbon tetrachloride and acetaminophen toxicity.[27,28]

Because of the possibility of sub-clinical injury, we used NAG excretion as a sensitive measure of tubular damage.[14] We noted 2 of 3 workers with chronically elevated NAG excretion in a pilot study.[3] In the present study, 4 of 15 workers had clinically elevated NAG excretion (greater than 4 mU/mg of creatinine) and 9 of 15 workers showed a greater than 25% increase in NAG excretion when compared to baseline. Intraindividual excretion of NAG will vary within 25%.[19,21] Most significant was that when DCP air exposure products exceeded 700 mg · min/m³ or when 3CNAC excretion exceeded 1.5 mg/day, NAG excretion over 24 h always was greater than for DCP exposures less than these cutoffs.

Stable baseline excretion of NAG was assumed to occur because exposure began following the weekend in most subjects and because NAG excretion will rapidly revert to normal when the injurious agent is removed. However, other pesticides, solvents, or unknown prior conditions may have affected the baseline and therefore the measurement of relative change. Other agents such as aminoglycosides, toxic metals, and underlying kidney disease are known to result in tubular enzyme leakage.[14] Such drugs and diseases were not indicated on questionnaires completed by these workers. It also must be noted that no complaints or cases of renal injury have been received by CDFA, and none are reported in the literature. Therefore, the significance of this finding is unclear with respect to renal sequelae in humans. It may represent a transient and reparable process. Because larger doses in animals have produced kidney necrosis, our finding may represent a low-dose effect. The positive dose-response across all workers and the abnormal increases in NAG excretion during DCP exposure in four workers attest to a potential cause-effect relationship.

Our studies show a firm relationship between DCP air exposure and 3CNAC excretion, as well as use of these measures to suggest that a subclinical nephrotoxicity in workers may be associated with DCP exposure.

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